Rhizobium nod Gene Inducers Exuded Naturally from Roots of Common Bean (Phaseolus vulgaris L.)[1]

Mariangela Hungria[2], Cecillia M. Joseph, and Donald A. Phillips*

Department of Agronomy & Range Science, University of California, Davis, California 95616

ABSTRACT

Four compounds exuded from young roots of a black-seeded bean (Phaseolus vulgaris L., cv PI165426CS) induce transcription of nod genes in Rhizobium leguminosarum biovar phaseoli. The three most active nod gene inducers were identified by spectroscopic methods (ultraviolet/visible absorbance, proton nuclear magnetic resonance, and mass spectrometry) as being eriodictyol (5,7,3',4'-tetrahydroxyflavanone), naringenin (5,7,4'-trihydroxyflavanone), and a 7-O-glycoside of genistein (5,7,4'-trihydroxyisoflavone). Comparisons with authentic standards verified the chemical structures of the aglycones and their capacity to induce β-galactosidase activity in R. leguminosarum strains containing nodA-lacZ or nodC-lacZ fusions controlled by R. leguminosarum biovar phaseoli nodD genes. Roots of 9-day-old seedlings released 42, 281, and 337 nanomoles per plant per day of genistein, eriodictyol, and naringenin, respectively. Genistein and naringenin induced higher maximum β-galactosidase activities and required lower concentrations for half-maximum induction than eriodictyol. Comparing the nod gene-inducing activity of seed rinses with root exudate from PI165426CS bean showed that root flavonoids were released at about 6% the rate of those from seeds on a molar basis, but on average the individual compounds from roots were approximately three times more active than nod gene inducers from seeds.

An initial stage of root nodule formation on legumes is triggered by plant flavonoids that induce transcription of the common nodulation genes nodABC in Rhizobium bacteria (reviewed in ref. 15). This process requires the presence of an appropriate gene product from the regulatory nodD gene family in rhizobia, which interacts with the flavonoid. Numerous flavonoid structures have been reported as natural nod gene inducers from various legumes, including anthocyanidins (10), chalcones (19, 22), flavanones (5, 28), flavones (20, 23), flavonols (10), and isoflavones (12).

Although nod-inducing flavonoids have been reported from roots or seeds of diverse legumes, a detailed comparison of active molecules released separately from both seeds and roots of the same legume is available only for alfalfa (Medicago sativa L.) (8, 19). In alfalfa, organ-specific differences are evident in the amounts of nod-inducing activity released and in the types of flavonoids present. Functional differences between alfalfa root and seed flavonoids are supported by their capacities to activate various NodD proteins (9) and by their differing effects on rhizobial growth rate (7). Whether other legumes show similar differences between nod gene-inducing flavonoids released from seeds and roots is unknown.

The capacity of a flavonoid to interact with a nodD gene product is strongly affected by its molecular structure (15). The nod gene inducers released from alfalfa are structurally different (flavones from seeds [8, 20]; a chalcone, a flavanone, and a flavone from roots [19]), but they appear similar enough to fit easily into a single active site on the NodD protein. In contrast, reports indicate that nod genes in R. leguminosarum bv[3] phaseoli are induced by both the isoflavone genistein, which was tested as a commercially available compound (3, 26), and by anthocyanidin and flavonol molecules, which were identified as natural inducers from bean seeds (10). Although anthocyanidins and flavonols are rather similar in structure, they differ markedly from genistein. If the NodD protein of R. leguminosarum bv phaseoli is activated by both flavonols and genistein, then beans may release nod gene inducer molecules with even simpler structures, such as monocyclic phenolics. The purpose of this study was to identify the major nod gene inducers exuded by sterile bean roots and to compare them with those released from seeds (10).

MATERIALS AND METHODS

Preparation of Exudates

Black bean seeds (Phaseolus vulgaris L., cv PI165426CS) (4) were sterilized and imbibed in aerated sterile water as described previously (10). The rinse solution was collected and replaced periodically for 48 h to provide seed exudates. After 48 h, 17 germinating seeds were placed on a stainless steel screen (4 mesh, 0.035 gauge) over a 473-mL plastic container filled with 300 mL of sterile, aerated, N-free nutrient solution (19). The container was covered with sterile clear plastic as roots developed into the nutrient solution. Growth chamber conditions supplied a 12/12 h light/dark cycle, 25/22°C, 50% RH, and a photosynthetic photon flux density (400–700 nm) of 320 μE m^{-2} s^{-1}. Solutions containing root exudates were changed and collected every 24 h for 8 d. The nod-inducing flavonoids identified in this study were purified

[3] Abbreviations: bv, biovar; δ_H, chemical shift of proton; 2H, two protons, etc.; C-1, carbon one, etc.; d, doublet; s, singlet; dd, double doublet; t, triplet; J, coupling constant; Hz, Hertz; I_{max}, the highest β-galactosidase activity; I_{50}, concentration for half-maximum induction.

from root exudates released by approximately 3400 plants between 216 and 240 h after imbibition. The solution from each container was centrifuged, filtered, and freeze dried (10).

Purification and Identification of nod Gene Inducers

Compounds were separated for initial nod-inducing assays by resuspending dried material from three containers in 10 mL of methanol:water (1:1, v/v). A 500-μL aliquot of the mixture was injected into an HPLC system equipped with a reverse-phase C-18 column and separated on a 70-min linear gradient from 0:99:1 to 99:0:1 (v/v) methanol:water:acetic acid followed by 20 min of isocratic chromatography with 99:1 (v/v) methanol:acetic acid.

Compounds were isolated for identification by eluting filtered root exudate from C-18 Maxi Clean cartridges and partitioning against an equal volume of hexane to remove lipids as described previously (10). Compounds were separated by semi-preparative HPLC, collected, and analyzed spectroscopically (UV/visible absorbance shift analyses, ^{1}H-NMR, and MS) (10). After tentative identifications, commercial standards were compared by spectroscopic analyses and co-chromatography. Standard interpretations were applied to UV/visible and ^{1}H-NMR spectra (6, 17, 18) and to MS analyses (6, 16). Flavonoids present in root exudates were quantified by integrating HPLC peaks (10), and values were corrected for losses during purification by quantifying recovery of known amounts of eriodictyol added to root exudate.

Biological Activities

The nod-inducing capacity of crude seed and root exudates, as well as specific compounds or fractions, was assayed as β-galactosidase activity transcribed from nodA-lacZ and nodC-lacZ plasmid fusions controlled by a R. leguminosarum bv phaseoli nodD gene (10). HPLC fractions were subdivided into portions equal to 1, 2, 5, 7.5, and 12.5% of the peak being tested and dried under vacuum for the assay. After compounds were identified, their nod gene-inducing activities were compared with HPLC-purified authentic commercial standards. Concentrations of flavonoids in assays were determined spectrophotometrically, using the following extinction coefficients (log ϵ): 4.50 at 263 nm in ethanol for genistein (27), 4.24 at 292 nm in methanol for eriodictyol (27), and 4.23 at 288 nm in ethanol for naringenin (17). Flavonoid standards were purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Inducing activities of putative and authentic compounds were compared for statistically significant effects of concentration by analysis of variance.

RESULTS

Release of nod Gene-Inducing Activity

Root exudate fractionated on the HPLC contained four nod gene-inducing factors active in rhizobial strain RBL1283 (nodA-lacZ) (Fig. 1A). Peak 1 showed a very low level of nod gene-inducing activity in the 1% dilution, but peaks 2 through 4 gave considerable activity (Fig. 1B). These results were confirmed with β-galactosidase assays in R. leguminosarum bv phaseoli, strains containing nodC-lacZ fusions, CE-3
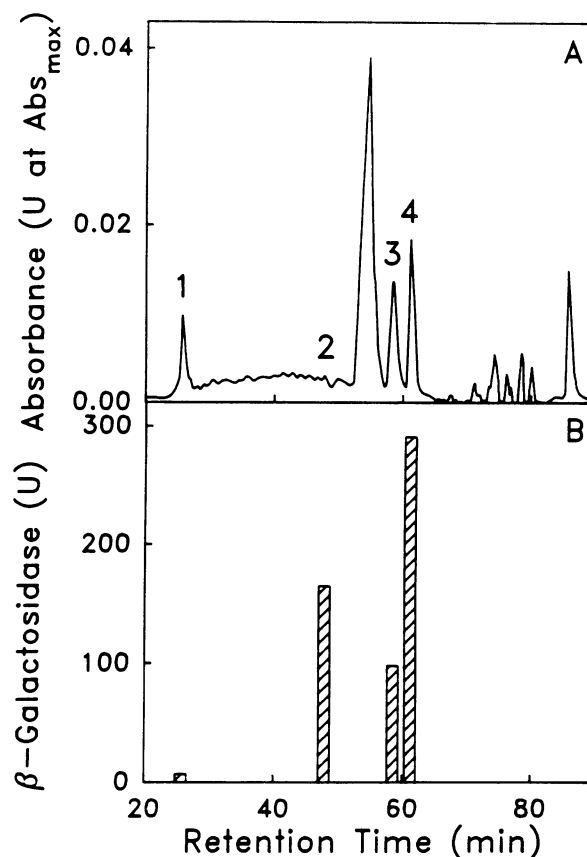

Figure 1. HPLC characteristics and nod-induction assays of bean root exudate. A, A_{max} (230–400 nm) of exudates fractionated on a reverse-phase C-18 column. B, β-Galactosidase activity induced from nodA-lacZ in rhizobial strain RBL1283 containing nodD from R. leguminosarum bv phaseoli. Assays contained 1% of the corresponding HPLC fraction.

(pA87) and 4292 (pIJ1737, pIJ1730), described previously (10) (data not shown). Emphasis in this study was placed on peaks 2 through 4, which contained the most active nod gene inducers in all assays.

Compound Identifications

Compound 2

Acid hydrolysis of peak 2 produced compound 2, which had UV/visible absorbance traits characteristic of an isoflavone (Table I). A bathochromic shift of band II with CH_3ONa indicated the presence of free hydroxyl groups on the A ring, and their positions were deduced to be at C-7 and C-5 by the shifts in band II with CH_3COONa and with $AlCl_3/HCl$, respectively. The absence of decomposition in CH_3ONa is characteristic of a single 4' hydroxyl on the B ring. These assignments of hydroxyls were supported by the following ^{1}H-NMR data for compound 2: δ_H ppm ([U-^{2}H]methanol): 8.05 (1H, s, C-2), 7.34 (2H, t, J = 17.7 Hz, C-6', C-2'), 6.83 (2H, d, J = 8.5 Hz, C-3', C-5'), 6.32 (1H, d, J = 1.8 Hz, C-8), 6.20 (1H, d, J = 1.8 Hz, C-6). MS data supported a mol wt = 270 and showed a major fragment at m/z 152. Thus, the data
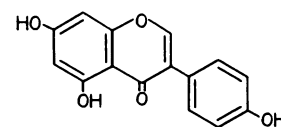
indicated a tentative identification of compound 2 as being genistein (5,7,4'-trihydroxyisoflavone) (Fig. 2). Peak 2 probably contains genistein glycosylated at the C-7 position because band II shows no shift in the presence of CH₃COONa (Table I).
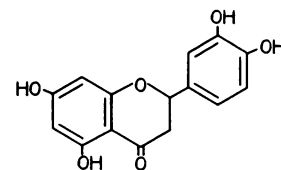
Compound 3

Peak 3 was designated as compound 3 because acid hydrolysis did not release a smaller molecule, alter HPLC retention time, change the UV/visible traits, or affect nod gene-inducing activity. Absorbance traits typical for a 5,7-dihydroxyflavanone were evident in a 44-nm bathochromic shift of band II with CH₃ONa (Table I). That interpretation was supported by a 35-nm shift in band II with CH₃COONa (7-hydroxyl) and a 19-nm shift with AlCl₃/HCl (5-hydroxyl). The ¹H-NMR data identified the following resonances: δ_H ppm ([U-²H]methanol): 6.83 (3H, d, J = 37.8 Hz, C-2', C-6', C-5'), 5.86 (2H, dd, J = 1.8, 6.1 Hz, C-8, C-6), 5.26 (1H, dd, J = 2.8, 12.8 Hz, C-2), 3.05 (1H, dd, J = 12.8, 17.1 Hz, C-3 trans), 2.67 (1H, dd, J = 3.0, 17.7 Hz, C-3 cis). MS data indicated a mol wt = 288 and gave fragments at m/z 287, 179, 153, 152, 136, and 123. Thus, the data supported a tentative identification of compound 3 as being eriodictyol (5,7,3',4'-tetrahydroxyflavanone) (Fig. 2).
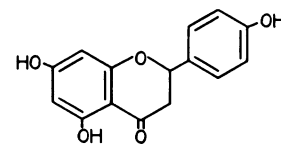
Compound 4

Peak 4 was designated as compound 4 because acid hydrolysis did not release a smaller molecule, alter HPLC retention time, change the UV/visible traits, or affect nod gene-inducing activity. A 5,7-dihydroxyl substitution pattern in compound 4 was supported by UV/visible shift traits similar to compound 3 (Table I). The ¹H-NMR data identified the following resonances: δ_H ppm ([U-²H]methanol): 7.30 (2H, d, J = 7.9 Hz, C-6', C-2'), 6.80 (2H, d, J = 8.5 Hz, C-3', C-5'), 5.86 (2H, d, J = 3.0 Hz, C-8, C-6), 5.33 (1H, dd, J = 1.8, 12.8 Hz, C-2), 3.11 (1H, dd, J = 3.6, 8.5 Hz, C-3 trans), 2.67 (1H, dd, J = 3.0, 17.1 Hz, C-3 cis). MS data indicated a mol wt = 272 with fragments at m/z 153, 152, 120, and 107. Thus, the data supported a tentative identification of compound 4 as being naringenin (5,7,4'-trihydroxyflavanone) (Fig. 2).

Figure 2. Structures of nod-inducing flavonoids exuded from bean roots. Genistein is released as a glycoside; eriodictyol and naringenin are exuded as aglycones.

Verification of Identifications

Spectroscopic analyses (UV/visible, ¹H-NMR, and MS) of authentic genistein, eriodictyol, and naringenin matched results obtained with compounds 2, 3, and 4, respectively. Furthermore, co-chromatography of standards with the root exudate compounds confirmed the putative identifications.

nod-Inducing Activities of Compounds

The nod gene-inducing activity of authentic and putative samples of compounds 2 through 4 showed similar concentration dependencies after repurification by HPLC immedi-

Table I. UV/Visible Absorbance Maxima (nm) of nod Gene Inducers in Bean Root Exudate
Standard reagents and procedures were used (17).

Compound[a]	Methanol	Shift Reagent				
		CH₃ONa	AlCl₃	AlCl₃/HCl	CH₃COONa	CH₃COONa/H₃BO₃
2G	261, 329sh[b]	271, 355sh	272, 309sh, 374	272, 309sh, 373	262, 330sh	261, 329sh
2A	261, 328sh	274, 326sh	273, 308sh, 371	273, 309sh, 371	272, 327	262, 335sh
3A	289, 323sh	245, 323	309, 376	308, 372	289sh, 324	290, 334sh
4A	289, 327sh	245, 323	311, 374	310, 371	284sh, 323	289, 331sh

[a] G, glycoside; A, aglycone. [b] sh, spectral shoulder.

Table II. *Comparison of nod-Inducing Activity for Putative Flavonoid Aglycones from Bean Roots and Authentic Standards*

Mean values are from three replicates assayed in rhizobial strain RBL1283.

Flavonoid Concentration	Genistein		Eriodictyol		Naringenin	
	Putative	Authentic	Putative	Authentic	Putative	Authentic
nM			units of *β-galactosidase*			
10	45	51	0	0	23	17
20	341	298	81	67	242	222
50	743	863	176	167	489	450
100	1390	1320	216	220	830	799
200	2290	2190	328	311	1210	1200
500	2900	2640	470	442	1530	1490
1,000	3010	3120	600	586	1960	2020
2,000	3960	3670	711	733	2460	2280
5,000	4000	3870	814	825	2480	2490
7,500	4120	4020	821	837	2500	2500
10,000	4180	4210	830	822	2410	2510
LSD 0.05	440		69		341	

ately before assaying (Table II). Comparisons among the flavonoids in root exudates showed that genistein had the highest I_{max} and the lowest I_{50}. No significant differences (P ≤ 0.05) in I_{max} values were detected for compound 2 (genistein) and unhydrolyzed peak 2 (genistein-7-*O*-glycoside). The I_{50} value for the glycoside, however, was slightly lower (120 *versus* 151 nM). Mean I_{50} values (nM) were genistein, 151; naringenin, 187; and eriodictyol, 270. Compounds 3 and 4, which are released as aglycones, induced similar amounts of $β$-galactosidase activity before and after hydrolysis.

Amounts of Compounds

Roots released total *nod* gene-inducing activity at about half the rate of seeds tested during the first 6 h of imbibition
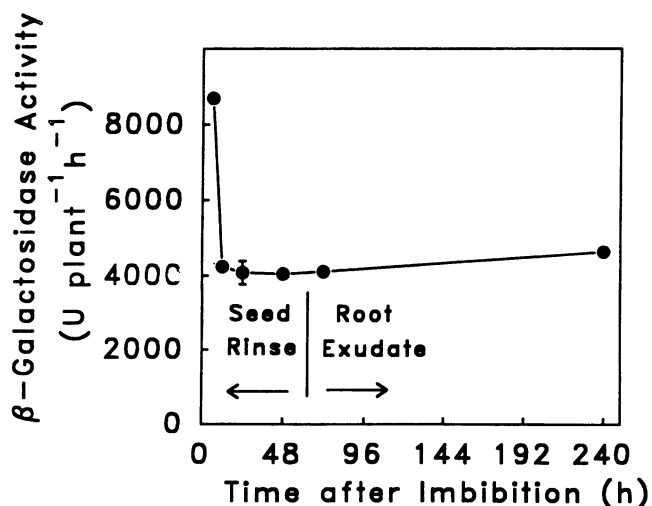
(Fig. 3). Using a 90% recovery rate measured as a correction factor for eriodictyol added to root exudate, HPLC integrations showed the following amounts of individual *nod*-inducing flavonoids exuded from bean roots between 216 and 240 h after imbibition (nmol plant^{-1} day^{-1}): genistein, 42; eriodictyol, 281; and naringenin, 337. The three root compounds showed an average I_{50} of 200 nM with a mean I_{max} of 2480 units (Fig. 4), whereas three flavonols and three anthocyanidins identified from seeds (10) gave a mean I_{50} of 670 nM and an average I_{max} of 1030 units.

DISCUSSION

Data from this study identify genistein, eriodictyol, and naringenin as being three *nod* gene-inducing flavonoids ex-

Figure 3. Release of *nod* gene-inducing activity from seeds and roots of bean line PI165426CS. Mean ± SE values are from six replicates assayed for $β$-galactosidase activity induced from *nodA-lacZ* controlled by *nodD* from *R. leguminosarum* bv *phaseoli* in rhizobial strain RBL1283.
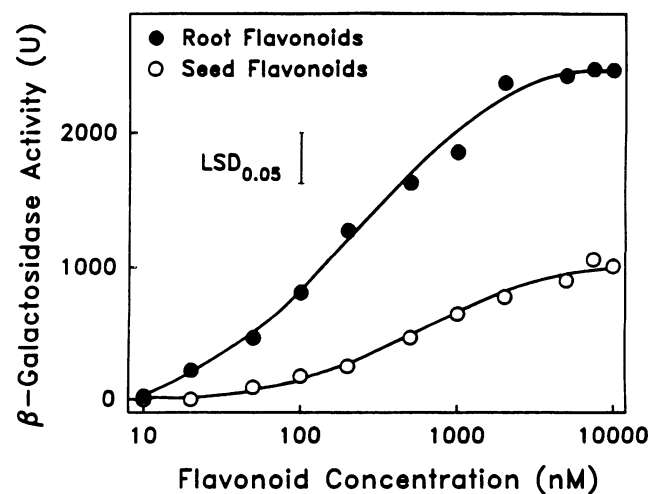

Figure 4. Induction of *nod* genes in rhizobial strain RBL1283 by flavonoids released from bean seeds and roots. Values for flavonoids identified as natural products from bean seeds (10) are means of six aglycones (delphinidin, petunidin, malvidin, myricetin, quercetin, and kaempferol). Root flavonoids are means of three *nod* gene inducers in root exudate identified in this study (genistein, eriodictyol, and naringenin). All means are from three replicates.

uded naturally from sterile bean roots. These aglycones are common plant flavonoids (2, 11), but they were not identified in bean seed rinses (10). Eriodictyol (5) and genistein (12) have been implicated previously as natural nod gene inducers released under sterile conditions from pea and soybean, respectively whereas naringenin has been used as a potent inducer in R. leguminosarum bv phaseoli (3, 26), its presence in natural bean exudates has not been reported.

Although the aglycones identified in this study were not observed in bean seed rinses (10), they share a common hydroxylation pattern with substitution at the C-4', C-5, and C-7 positions. Whereas the 4'-hydroxyl may contribute to activity, it clearly is not required, because chrysin (5,7-dihydroxyflavone) and galangin (3,5,7-trihydroxyflavone) induce nod-lacZ transcription in R. leguminosarum bv phaseoli (3). Moreover, the aryl shift of the B ring from the C-2 to the C-3 position in genistein (Fig. 2) places the 4'-hydroxyl of that compound in a much different position than for any of the other natural nod gene inducers from bean. It seems reasonable, therefore, that hydroxylation similarities in these compounds may reflect their common biosynthetic pathways (24) rather than uniform requirements for interacting with the nodD gene product.

One notable feature of bean is the large variety of flavonoid structures that is released as natural nod gene inducers. To the anthocyanins and flavonols released from seeds (10), one must now add the flavanones and isoflavone identified in root exudate (Fig. 2). These diverse compounds all were active with both the nodA-lacZ and the nodC-lacZ fusions tested in this study, even though nodA and nodC are transcribed separately in R. leguminosarum bv phaseoli (26). Natural nod gene inducers released from alfalfa seeds and roots show clear structural differences (5-hydroxy from seeds versus 5-deoxy from roots; 8, 19), but no such distinction is evident for bean. One cannot make definitive statements about interactions between flavonoids and nodD gene products until those purified proteins are available for binding studies, but the diversity of active flavonoid structures released from bean and their uniformly higher I_{50} values suggest that NodD proteins from R. leguminosarum bv phaseoli are much less specific for flavonoids than their counterparts in R. meliloti (8, 10, 19). Despite this possibly lower level of specificity in the R. leguminosarum bv phaseoli NodD proteins, no nod gene-inducing monocyclic phenolics, comparable to those isolated from wheat root extracts (14), were identified in bean root exudates. The suggestion that structural differences in bean nod gene inducers may be related to their capacity to inhibit nod gene expression in other rhizobia (10) is supported by effects of bean root flavonoids on other rhizobial species (e.g. genistein inhibits nod gene induction in R. leguminosarum bvs viceae and trifolii [5]; naringenin inhibits nod gene induction in R. meliloti [21] and some Bradyrhizobium japonicum strains [13]).

Data on the amount of flavonoids released from germinating bean seeds (10) and roots (Fig. 2) also offer an interesting comparison with recently described nod gene-inducing flavonoids that are released from alfalfa (8, 19, 20). Bean seeds used for these studies were about 80 times larger than alfalfa seeds, but the rates of discharge for nod gene inducers identified from bean are 6000 to 7000 times higher than the comparable

values for alfalfa (bean: seeds, 497 nmol seed^{-1} h^{-1} [10], roots, 28 nmol plant^{-1}·h^{-1}; alfalfa: seeds, 82 pmol seed^{-1} h^{-1} [8], roots, 4 pmol plant^{-1} h^{-1} [19]). These numerical comparisons must be tempered with the knowledge that they are based on a limited number of plant and bacterial genotypes, but such large differences in total amount of flavonoids might be explained by several factors. First, beans may release larger quantities of flavonoids than alfalfa because the bean nod gene inducers have higher I_{50} values than those of alfalfa when tested in their respective rhizobia (e.g. genistein I_{50} = 150 nM; 4,4'-dihydroxy-2'-methoxychalcone I_{50} = 1 nM [19]). Second, due to its annual life history, bean may benefit more from an early establishment of root nodules than the perennial alfalfa.

Overall, bean and alfalfa root exudates contain lesser quantities of nod gene-inducing flavonoids than seed rinses (Fig. 3) (8, 19), but the average activity of the individual root compounds is greater than that of nod gene inducers from seed rinses (Fig. 4) (8, 19). This fact suggests that soil surrounding these organs should be viewed as separate ecochemical zones in terms of both the amounts and structures of flavonoids. With the identity of nod gene-inducing flavonoids established for seeds (10) and roots (Fig. 2), other biological effects of these natural compounds can be examined. Although it has been reported that R. leguminosarum bv phaseoli shows a positive chemotaxis toward luteolin (1), tests can now be conducted with the flavonoids that are actually present in the seed and root zone of bean. Similarly, bean flavonoids can be tested for effects comparable to those demonstrated for alfalfa flavonoids on growth of rhizobia (7) and germination of Glomus (25). Testing these possible roles of naturally released bean flavonoids can help define ecochemical differences between seed and root zones.

LITERATURE CITED

1. **Aguilar JMM, Ashby AM, Richards AJM, Loake GJ, Watson MD, Shaw CH** (1988) Chemotaxis of Rhizobium leguminosarum biovar phaseoli towards flavonoid inducers of the symbiotic nodulation genes. J Gen Microbiol **134:** 2741–2746
2. **Bohm BA** (1975) Flavanones and dihydroflavonols. In JB Harborne, TJ Mabry, H Mabry, eds, The Flavonoids. Academic Press, New York, pp 560–631
3. **Davis EO, Johnston AWB** (1990) Regulatory function of the three nodD genes of Rhizobium leguminosarum biovar phaseoli. Mol Microbiol **4:** 933–941
4. **Dickson MH, Petzoldt R** (1988) Deleterious effects of white seed due to p gene in beans. J Am Soc Hortic Sci **113:** 111–114
5. **Firmin JL, Wilson KE, Rossen L, Johnston AWB** (1986) Flavonoid activation of nodulation genes in Rhizobium reversed by other compounds present in plants. Nature **324:** 90–92
6. **Grayer R** (1989) Flavanoids. In PM Dey, JB Harborne, eds, Plant Phenolics. Academic Pres, London, pp 283–323
7. **Hartwig UA, Joseph CM, Phillips DA** (1991) Flavonoids released naturally from alfalfa seeds enhance growth rate of Rhizobium meliloti. Plant Physiol **95:** 797–803
8. **Hartwig UA, Maxwell CA, Joseph CM, Phillips DA** (1990) Chrysoeriol and luteolin released from alfalfa seeds induce nod genes in Rhizobium meliloti. Plant Physiol **92:** 116–122
9. **Hartwig UA, Maxwell CA, Joseph CM, Phillips DA** (1990) Effects of alfalfa nod gene-inducing flavonoids on nodABC transcription in Rhizobium meliloti strains containing different nodD genes. J Bacteriol **172:** 2769–2773
10. **Hungria M, Joseph CM, Phillips DA** (1991) Anthocyanidins and flavonols, major nod-gene inducers from seeds of a black-seeded common bean (Phaseolus vulgaris L.). Plant Physiol **97:** 751–758

11. **Ingham JL** (1983) Naturally occurring isoflavonoids (1855–1981). Fortschr Chem Org Naturst **43**: 1–266

12. **Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER** (1987) Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavone isolated from *Glycine max.* Proc Natl Acad Sci USA **84**: 7428–7432

13. **Kosslak RM, Joshi RS, Bowen BA, Paaren HE, Appelbaum ER** (1990) Strain-specific inhibition of *nod* gene induction in *Bradyrhizobium japonicum* by flavonoid compounds. Appl Environ Microbiol **56**: 1333–1341

14. **Le Strange KK, Bender GL, Djordjevic MA, Rolfe BG, Redmond JW** (1990) The *Rhizobium* strain NGR234 *nodD1* gene product responds to activation by the simple phenolic compounds vanillin and isovanillin present in wheat seedling extracts. Mol Plant-Microbe Interact **3**: 214–220

15. **Long SR** (1989) Rhizobium-legume nodulation: life together in the underground. Cell **56**: 203–214

16. **Mabry TJ, Markham KR** (1975) Mass spectrometry of flavonoids. *In* JB Harborne, TJ Mabry, H Mabry, eds, The Flavonoids. Academic Press, New York, pp 78–126

17. **Mabry TJ, Markham KR, Thomas MB** (1970) The Systematic Identification of Flavonoids. Springer-Verlag, New York

18. **Markham KR, Mabry TJ** (1975) Ultraviolet-visible and proton magnetic resonance spectroscopy of flavonoids. *In* JB Harborne, TJ Mabry, H Mabry, eds, The Flavonoids. Academic Press, New York, pp 45–77

19. **Maxwell CA, Hartwig UA, Joseph CM, Phillips DA** (1989) A chalcone and two related flavonoids released from alfalfa roots induce nod genes of *Rhizobium meliloti.* Plant Physiol **91**: 842–847

20. **Peters NK, Frost JW, Long SR** (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233**: 977–980

21. **Peters NK, Long SR** (1988) Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol **88**: 396–400

22. **Recourt K, Schripsema J, Kijne JW, Van Brussel AAN, Lugtenberg BJJ** (1991) Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* biovar *viciae* results in release of *nod* gene activating flavanones and chalcones. Plant Mol Biol **16**: 841–852

23. **Redmond JW, Batley M, Djordjevic MA, Innes RW, Kuempel PL, Rolfe BG** (1986) Flavones induce expression of nodulation genes in *Rhizobium.* Nature **323**: 632–635

24. **Stafford HA** (1990) Flavonoid Metabolism. CRC Press, Boca Raton

25. **Tsai SM, Phillips DA** (1991) Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl Environ Microbiol **57**: 1485–1488

26. **Vázquez M, Dávalos A, de las Peñas A, Sánchez F, Quinto C** (1991) Novel organization of the common nodulation genes in *Rhizobium leguminosarum* bv. *phaseoli* strains. J Bacteriol **173**: 1250–1258

27. **Weast RC,** ed (1969) Handbook of Chemistry and Physics. The Chemical Rubber Co, Cleveland

28. **Zaat AJ, Schripsema J, Wijffelman CA, Van Brussel AAN, Lugtemberg BJJ** (1989) Analysis of the major inducers of the *Rhizobium nodA* promoter from *Vicia sativa* root exudate and their activity with different *nodD* genes. Plant Mol Biol **13**: 175–188